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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
PATENT EXAMINING OPERATION

Attorney Docket No.: 113737.6

Assistant Commissioner for Patents  
**BOX PATENT APPLICATION**  
Washington, D.C. 20231

**NEW APPLICATION TRANSMITTAL**  
**UNDER 35 U.S.C. 371**

Transmitted herewith for filing is the patent application of

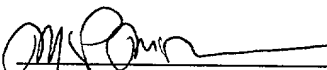
Inventors: Ulrich Zimmermann and Marcus Behringer  
For: Process for Obtaining Highly Purified Alginates

Please direct all correspondence to:

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Respectfully submitted,

Telephone Number: (215) 981-4442  
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Matthew P. McWilliams  
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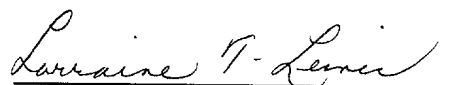
Dated: February 13, 2001

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Date of Deposit February 13, 2001

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Lorraine T. Lewis  
(Typed or printed name of person mailing paper)

  
(Signature of person mailing paper or fee)

Practitioner's Docket No. 113737.6

PATENT

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ulrich Zimmermann and Marcus Behringer

Serial No.: N/A

Group No.: N/A

Filed: herewith

Examiner: N/A

For: PROCESS FOR OBTAINING HIGHLY PURIFIED ALGINATES

BOX

Assistant Commissioner for Patents

Washington, D.C. 20231

## PRELIMINARY AMENDMENT TRANSMITTAL

1. Transmitted herewith is a preliminary amendment for this application.

## STATUS

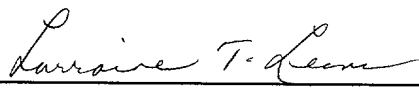
2. Applicant is  
☒ a small entity.  
☐ other than a small entity.

Express Mailing Label No. EL715949793USDate of Deposit February 13, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, BOX PATENT APPLICATION, Washington, DC 20231.

Lorraine T. Lewis

(Typed or printed name of person mailing paper)



(Signature of person mailing paper or fee)

## EXTENSION OF TERM

3. The proceedings herein are for a patent application and the provisions of 37 C.F.R. 1.136 apply.

*(complete (a) or (b), as applicable)*

- (a) ☐ Applicant petitions for an extension of time under 37 C.F.R. 1.136  
(fees: 37 C.F.R. 1.17(a)(1)-(4)) for the total number of months checked below:

	Extension (months)	Fee for other than small entity	Fee for small entity
<input type="checkbox"/>	one month	\$ 110.00	\$ 55.00
<input type="checkbox"/>	two months	\$ 390.00	\$ 195.00
<input type="checkbox"/>	three months	\$ 890.00	\$ 445.00
<input type="checkbox"/>	four months	\$ 1,390.00	\$ 695.00
<input type="checkbox"/>	five months	\$ 1,890.00	\$ 945.00

Fee: \$ \_\_\_\_\_

If an additional extension of time is required, please consider this a petition therefor.

*(check and complete the next item, if applicable)*

- ☐ An extension for \_\_\_\_\_ months has already been secured. The fee paid therefor of  
\$ \_\_\_\_\_ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request \$ \_\_\_\_\_

**OR**

- (b) ☒ Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition for extension of time.

### FEE FOR CLAIMS

4. The fee for claims (37 C.F.R. 1.16(b)-(d)) has been calculated as shown below:

(Col.1)		(Col. 2)	(Col. 3)	SMALL ENTITY			OTHER THAN A SMALL ENTITY		
Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Addit. Fee	OR	Rate	Addit. Fee	
Total	* Minus	**	=	x \$9 =	\$		x \$18 =	\$	
Indep.	* Minus	***	=	x \$40	\$		x \$80 =	\$	
[ ] First Presentation of Multiple Dependent Claim				+ \$135 =	\$		+ \$270 =	\$	
				Total Addit. Fee	\$ _____	OR	Total Addit. Fee	\$ _____	

- (c) ☒ No additional fee for claims is required.

OR

(d) ☐ Total additional fee for claims required \$ \_\_\_\_\_.

**FEE PAYMENT**

5. ☐ Attached is a check in the sum of \$ \_\_\_\_\_.  
☐ Charge Account No. 50-0436 the sum of \$ \_\_\_\_\_.  
A duplicate of this transmittal is attached.

**FEE DEFICIENCY**

6. ☒ If any additional extension and/or fee is required, charge Account No. 50-0436.

**AND/OR**

☒ If any additional fee for claims is required, charge Account No. 50-0436.

  
\_\_\_\_\_  
SIGNATURE OF PRACTITIONER

Reg. No. 46,922

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Practitioner's Docket No. 113737.6

*PATENT*

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of: Ulrich Zimmermann and Marcus Behringer

Serial No.: N/A

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Examiner: N/A

For: Process for Obtaining Highly Purified Alginates

Assistant Commissioner for Patents  
Washington, D.C. 20231

**PRELIMINARY AMENDMENT AND REMARKS**

Sir:

In the application transmitted herewith, Applicant requests entry of the following preliminary amendment and remarks.

09763350-044304

## AMENDMENTS

### IN THE SPECIFICATION

At page 1, line 4, please insert;

--FIELD OF THE INVENTION--

At page 1, line 9, please insert;

--BACKGROUND OF THE INVENTION--

At page 3, line 35, please insert;

--Coating materials of alginates are also known from WO-A-93/24077. The process therein presented already starts from alginates which are commercially available. This alginate preparation is treated with a complexing agent to remove the divalent metal ions. The subsequent treatment with activated charcoal absorbs the polyphenols still present together with the proteins and fucose units attached thereto. After the treatment with activated charcoal the alginate is precipitated from the solution, washed and filtered to remove further impurities. The molecular weight of said alginates is located in the lower range, it is in the range of about 2 to 300 kD.

Alginates rich of mannuronic acids are known from Biomaterials, volume 8, 1997, pages 707 to 713. Said alginate is extracted from brown algae and subsequently purified. The purification process is based on the extraction of alginate beads cross-linked with  $Ba^{2+}$  ions (produced from coal-treated raw algine extract) with weak acids, citrate and ethanol. Then a dialysis is made, whereafter the alginate is then precipitated with ethanol.--

At page 4, line 5, please insert;

--SUMMARY OF THE INVENTION--

AT PAGE 4, LINE 15, PLEASE DELETE;

[This objective is achieved by a process and an alginate composition with the features according to Patent Claim 1 and 16 respectively. Advantageous embodiments and applications of the invention are derived from the dependent claims.]

At page 11, line 25, please insert;

--BRIEF DESCRIPTION OF THE DRAWINGS--

At page 12, line 3, please insert;

--DETAILED DESCRIPTION OF THE INVENTION--

PHLEGAL: #973966 v1 (KV\$M01!.DOC)

## **IN THE CLAIMS**

Please cancel claims 1 through 28 without prejudice or disclaimer.

Please insert the following new claims;

29. A process for obtaining a highly-purified alginate composition, the process comprising the steps of:
  - a) extracting an algae material in a solution with a complex forming agent,
  - b) filtering the solution,
  - c) precipitating an alginate out of the solution,
  - d) collecting and dewatering the precipitated alginate, and
  - e) repeating the steps a) to d) at least once.
30. A process according to Claim 29, wherein ethylene diamine tetra acetic acid is used as a complex forming agent for the extraction.
31. A process according to Claim 29, wherein the extracting takes place in a soda solution.
32. A process according to Claim 30, wherein activated carbon is added for the extraction of the solution.
33. A process according to Claim 29, wherein, before the filtering of the solution, sedimentation of cell constituents and particles from the solution is carried out with a porous binding agent.
34. A process according to Claim 33, wherein the sedimentation takes place with a porous granulate on the basis of diatomaceous earth, cellulose, or recycling materials from regenerated raw materials.
35. A process according to Claim 29, wherein the filtering takes place with deep filters, and the solution is subjected to more than one filtration, and the pore size of the filters used decreases for successive filtrations.
36. A process according to Claim 29, wherein the precipitation of the alginate takes place in a solution containing ethanol.



37. A process according to Claim 36, wherein the ethanol content is selected in the range from about 10% to about 50%.
38. A process according to Claim 29, wherein the collecting of the precipitated alginate is effected by foaming out of the solution, by decanting from the solution, or by stirring the solution with a stirring and collecting device.
39. A process according to Claim 29, wherein the dewatering of the alginate takes place at room temperature.
40. A process according to Claim 29, wherein the algae material used in the process is fresh algae material occurring in nature, fresh algae material cultivated in a bioreactor or tank system, or algae material from fusioned or regenerated algae cells.
41. A process according to Claim 29, wherein the algae material used in the process is specific organ or tissue parts of algae or algae parts, or specific organ or tissue parts of algae or algae parts from specific stages of the development cycle of algae.
42. A process according to Claim 29, wherein the algae material used in the process is brown algae or other alginate-producing fresh-water or salt-water algae.
43. An alginate composition comprising a mixed polymer of mannuronic acid and guluronic acid, in which the ratio of mannuronic acid to guluronic acid in the mixed polymer is in the range from about 0.1 to about 9, and the mean molecular weight of the mixed polymer is greater than about 350 kD.
44. An alginate composition according to Claim 43, wherein an aqueous solution of the alginate composition at approximately a 0.1% concentration, has a viscosity in the range from about 10 to about 15 mPa s.
45. An alginate composition according to Claim 43, wherein an aqueous solution of the alginate composition at approximately a 0.5 % concentration, has a viscosity in the range from about 250 to about 300 mPa s.

46. An alginate composition according to Claim 43, wherein an aqueous solution of the alginate composition, under illumination with an excitation wavelength of 366 nm in the spectral range from 380 to 550 nm, does not show any fluorescence emission.
47. An alginate composition according to Claim 43, wherein the alginate composition under colour tests with the Folin-Denis reagent or with dimethocycbenzaldehyde, does not show any colouration.
48. An alginate composition according to Claim 43, wherein an aqueous solution of the alginate composition, under illumination with an excitation wavelength of 270 nm in the spectral range from 300 to 500 nm, does not show any fluorescence emission.
49. An alginate composition according to Claim 43, wherein the alginate composition does not contain any proteins detectable with the photometric protein test according to Bradford.
50. Alginate composition according to Claim 43, wherein the alginate composition does not initiate any significant immunological reaction when implanted in the kidneys of BB/OK rats.
51. An alginate composition according to Claim 43, wherein the application of the XTT/MTT test, or the cell rotation method, or an electrical cell number and cell size analysis, does not lead to any detectable lymphocyte activation.
52. An alginate composition according to Claim 43, manufactured by the process according to Claim 29.
53. An alginate compound according to Claim 43, for use in the manufacture of alginate capsules or coatings for transplant surgery and for other medical applications and for the food industry.
54. An alginate compound according to Claim 43, for use in the manufacture of alginate capsules or coatings for allogenic and xenogenic tissue.

55. An alginate compound according to Claim 43, for use in the manufacture of alginate capsules or coatings for Islets of Langerhans, parathyroid gland or epithelial body tissue, endocrine tissue, or dopamine-excreting cells.

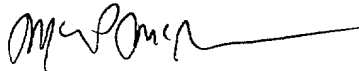
## REMARKS

All amendments submitted with this preliminary amendment find support in the original specification and claims. Thus no new matter has been added. The specification has been amended to more completely describe the invention. The claims have been amended to remove multiple dependent claims and to better describe the claimed invention. No new claims have been added over the number in the original application. Applicant respectfully request entry of the amendments submitted herewith.

Applicant's undersigned agent may be reached in our Philadelphia Office at (215) 981-4442 (fax: (215) 981-4271). All correspondence should be directed to the address shown below.

Respectfully Submitted,

PEPPER HAMILTON LLP



Matthew McWilliams  
Registration No. 46,922

Date: February 13, 2001

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14720/US Dr/hr

PROCESS FOR OBTAINING HIGHLY-PURIFIED ALGINATES

- 5 The invention relates to a process for obtaining highly-purified alginates, in particular from brown algae, and alginates manufactured with this process with a high degree of polymerisation, and the use thereof.
- 10 Alginates have a large number of applications in the food technology sector (e.g. Askar in "Alimenta", Vol. 21, 1982, p. 165 ff.) and in textile technology, and increasingly in the pharmaceutical sector, medicine, biochemistry, and biotechnology. The alginates acquired according to the processes
- 15 known hitherto from algae plants (for an overview, see for example D.J. McHugh: "Production and Utilization of Products from Commercial Seaweeds" in "FAO Fisheries Technical Papers", Vol. 288, 1987, Chap. 2) are characterised by fluctuations in composition and structure, as well as by impurities. This
- 20 derives from the fact that the raw alginate is extracted from biomass which is acquired from the wild population. The algae populations which grow in coastal waters in particular are subjected to numerous geographical, seasonal, and substance influences (environmental pollution). Added to this is the fact
- 25 that, during the harvest, the algae may be collected together with foreign substances, and are subjected to chemical treatment for preservation and decolouration (e.g. with formalin and/or hypochloride).
- 30 The raw alginates available hitherto are therefore mixed polymers of variable structure with impurities, which may include in particular toxic chemicals. In view of the fact that in food and textile technology interest pertains in particular in the formative properties of the alginates, processes are
- 35 being developed for the subsequent purification of the raw alginates, as explained hereinafter, in the first instance for biological-medical applications.

Processes for the purification of alginates (such as are available, for example, from the company Keltone LV or Kelco Nutrasweet) are described, for example, in DE-OS 4 204 012, US-A 5 429 821 (or US-A 5 656 468) and in the publication by P. De Vos et al. in "Diabetologia" (Vol. 40, 1997, p. 262 ff.). These processes generally have the disadvantage of a high expenditure of energy (applications of electrophoresis, freeze-drying, or centrifuging, heating, or boiling), a high environmental burden (use of acids such as HCl, H<sub>2</sub>SO<sub>4</sub>, non-biodegradable solvents such as CHCl<sub>3</sub>, or heavy metal ions such as barium, lead, or cadmium), and a restriction on the attainable mean molecular weight of the purified final material. Further disadvantages of the conventional processes are derived individually from the following explanations:

With the process known from DE-OS 42 04 012, a raw alginate solution is subjected to treatment with a complex forming agent, acid extraction at high temperatures (about 70 °C), washing, treatment with concentrated alcohol (about 80 %), and further treatment with a complex forming agent. A dialysis process and freeze drying follows (or electrophoresis or centrifuging) to obtain the purified alginate. This process is disadvantageous because of the high energy expenditure, the large number of process stages, the use of toxic materials (e.g. barium as the complex forming agent) and the restriction to alginates (< 500 kD). One special problem, however, is that the purification effect of this process is only restricted.

In DE-OS 42 04 012 the purified alginates are indeed referred to as nitrogen-free substances, but only on the assumption of freedom from mitogens. It has however been shown that the animal experiments developed in the 'eighties and implemented in DE-OS 42 04 012, are not suitable for demonstrating freedom from mitogens, such as would fulfil the requirements of modern biomedical applications, such as implant technology. Thus, for example, the animal experiments with the conventional purification process were carried out with what are known as Lewis rats. In the interim, however, it has been proved by

G. Klöck et al. in "Biomaterials" (Vol. 18, 1997, p. 707 ff.) and by P. Gröhn (Dissertation at the University of Würzburg, 1998) that Lewis rats have relatively low sensitivity to nitrogenic substances. Implanted alginate capsules, which did not induce any inflammation reactions in Lewis rats after three weeks, led, for example with what are referred to as BB-OK rats ("Bio Breeding / Ottawa Karlsburg"), to inflammation reactions. It follows from this that the nitrogen-free substances known from DE-OS 42 04 012 cannot in fact be regarded as highly-purified, and are only capable of restricted use due to reduced biocompatibility in biomedical applications.

With the purification processes known from US-A 5,429,821 and US-A 5,656,468, an acid precipitation is likewise carried out. Again, high-temperature process stages are required, and, for the final acquisition of the alginates, centrifuging, electrophoresis, or freeze-drying. The alginates acquired with this process are restricted to molecular weights of less than 200 kD. Drying follows at 80 °C, during which drying artefacts may be incurred in the mixed polymer due to structural or substance changes.

Another problem arises from the restriction to relatively low molecular weights. Thus, for example, it is known from "Immobilized Enzymes" by J. Chibata (A Halsted Press Book, John Wiley & Sons, 1978) that the bio-toxicity of materials decreases with the increase of the molecular weight.

In the final analysis, the process referred to heretofore according to P. de Vos et al. is also characterised by numerous process stages, including acid precipitation, the use of toxic chemicals (chloroform), the use of highly-concentrated ethanol (about 70 %), and the use of centrifuging, electrophoresis, or freeze-drying.

A further disadvantage of all purification processes consists of their restriction to the purification of commercially available raw alginates. The processes are not applicable to fresh

material or harvested biomass. In addition to this, the processes are not practicable for large-scale technical application because of the elaborate process performance, the energy expenditure, and the use of toxic chemicals.

5

The objective on which the invention is based is of providing a process for the obtaining of highly-purified alginates with which the disadvantages of conventional purification processes are avoided and the manufacture of highly-purified alginates is rendered possible, in particular in large-scale technical production. The objective of the invention is also to provide a new type of alginate, in particular with a higher molecular weight and increased viscosity than conventional alginates.

10

15

This objective is achieved by a process and an alginate composition with the features according to Patent Claim 1 and 16 respectively. Advantageous embodiments and applications of the invention are derived from the dependent claims.

20

The term "highly-purified alginate" is to be understood in this context to be an alginate composition which is capable of reproducible manufacture, which features predetermined molecular weight and/or viscosity parameters, and high sterility, purity, and biocompatibility, the latter feature relating, for example, to the fact that alginates which are highly purified in accordance with the invention do not induce any, or negligibly low, foreign body reaction in auto-immune diabetic BB/OK rats, even after several weeks of implantation.

25

30

By contrast with the conventional purification processes, which are all adapted to commercially available raw alginates, but which represent blends or mixtures of different algae materials, with the inclusion of animal or other foreign materials, and therefore cannot in principle provide highly-purified alginate, according to the invention alginate manufacture or acquisition is achieved, which derives predominantly from clean fresh algae material or dried algae material as the source material.

35

Provision is made in particular for the algae material to be



5 treated initially in the presence of complex forming agents,  
whereby sedimentation of cell constituents and particles takes  
place with a binding agent in the form of a granulate or a  
comparable porous material. After a filtration process, a  
precipitation stage takes place, for preference under  
simultaneous blowing in of a carrier gas, under the effect of  
which the precipitated alginate floats on the solution. This  
foaming (floating) is not mandatorily required. Precipitated  
alginate can also be separated from the solution by other means  
10 (e.g. by a decanting process). The alginate acquired in this way  
can be drawn off from the surface of the solution, or dehydrated  
in air or by a filter press as a residue after decanting.  
Depending on the application, this purification process, or part  
steps of this purification process, are carried out once or  
15 several times. After the last purification, subsequent washing  
and air drying takes place.

20 This procedure has the following advantages. During the  
acquisition of the alginate, toxic chemicals and high-  
temperature conditions can be entirely done without. The  
individual process stages are based on inherently known and  
easily mastered techniques, the combination of which according  
to the invention is particularly advantageous with regard to  
process costs, material investment, and process speed. For the  
25 first time, by contrast with all conventional attempts at  
purification, bio-compatibility is achieved of the alginate  
material acquired, so that the area of application of this  
material is substantially increased, especially in the fields of  
biology and medicine. The biocompatible product manufactured  
30 according to the invention is based exclusively on alginate. The  
alginate is free of additives, such as, for example, immuno-  
suppressants or immune-stimulating substances or phenols or  
compounds similar to phenols, and can be used directly in this  
condition.

35 The stages of the process can be carried out without any  
problems on a relatively small scale, e.g. at the place of the  
algae harvesting, or can also be carried out on a technically

large scale. By specific control of the precipitation reaction, impurities caused by fucoidan will be avoided, with the result that the purity of the alginate acquired improves in comparison with conventional alginates.

5

The direct processing of alginate material provides a series of advantages. Firstly, disadvantages with conventional algae harvests, which are caused by decomposition and rotting at the place of harvesting, and by preservative treatment with chemicals, can be avoided completely. Secondly, the harvested algae can be separated according to organs or tissue sections, before the algae acquisition is carried out. Because the different algae tissues differ due to a variety of conditions of the monomer mannuronic acid and guluronic acid, specific highly-purified alginates can be manufactured with a specific mannuron-guluron composition. The same applies accordingly to the choice of specific tissue parts for the achievement of a specific molecular weight. It is possible for alginate compositions according to the invention to be acquired with a molecular weight of more than 1,000 kD. In the final analysis, the process according to the invention can be achieved without elaborate centrifuge stages, as a result of which the practical implementation is further simplified. The specific acquisition of different types of high-purity alginate (e.g. alginate rich in guluronate and mannuron) can be achieved by the specific acquisition of specific algae species (Laminariales, Ectocarpales, Fucales, and other algae species containing alginate), which differ in the chemical structure of the particular alginate concerned. With the organ-specific selection, by contrast, phylloids, cauloids, and rhizoids are separated from the algae and processed separately. This differentiation can be further refined in that individual tissues are separated from the different organs, and the alginate is acquired separately from these tissues.

35

The invention is particularly well-suited to the acquisition of alginate from fresh or dried algae material (in particular brown algae). It is however also possible, with the process according

to the invention, for commercially available raw alginates to be purified or for the process according to the invention to be combined with specific washing or separation stages (e.g. centrifuging), which are known from the conventional purification process.

Advantages of the invention are derived from the fact that the high-purity alginate is acquired directly from the algae plants under control and supervision of all the process stages. Toxic chemicals (solvents in particular) are avoided, so that use for pharmaceutical purposes is possible without further ado. The foaming of the precipitated alginate represents a particularly simple and low-energy separation process.

According to a preferred embodiment of the invention, the following process stages are carried out initially on fresh algae material or dried material, and then partially repeated on highly-purified alginate, which was acquired from the first or earlier process sequences. The algae material or commercial alginate is designated hereinafter as "source material".

According to the invention, the source material is initially extracted in the presence of complex forming agents, if appropriate in a soda solution (see below, Example 1).

Subsequently, cell constituents and particles present in the solution are brought to sedimentation by the addition of a granulate and, if required, by the addition of ion exchangers (such as, for example, amberlite), and the solution is then filtered. The sedimentation can, as an alternative, be carried out with the use of electrographite (e.g. in globular form). In this situation, electrographite is introduced into the solution and charged by means of a current flow between two electrodes suspended in the solution. The charged electrographite globules show an accumulation of impurities, which in this case comprise the cell constituents and foreign particles concerned. The sedimentation is used for preference in the purification of commercial algae material, but can also be dispensed with entirely, depending on the application (see below, Example 2).

The filter stage may comprise a multiple filtering process with pore sizes being reduced step by step, e.g. from 15  $\mu\text{m}$  to 0.1  $\mu\text{m}$ . From the filtered solution, the alginate is precipitated through a suitable precipitation medium. The precipitation is carried out for preference with an alcohol (e.g. ethanol). An acid or another suitable precipitation agent can, however, also be used. The alcohol concentration is selected in the range between 10 % to 50 %, for preference in the range from 30 % to about 50 %. In this concentration range impurities due to immunologically active polysaccharides such as fucoidan remain in the solution, and can therefore be separated from the alginate. In the event of higher alcohol concentrations being used, such as in the process according to De Vos et al., these undesirable polysaccharides cannot be separated. During the precipitation, for preference, the solution flows through with a propellant gas (e.g. air). The precipitated alginate is driven upwards by the air which is blown in, and can be easily separated from the surface of the solution by a suitable device (e.g. net, sieve, or the like). The alginate collected can then be dewatered with a filter press.

The process stages referred to are repeated, depending on the application, either in whole, in part, or in a partially modified form. After the last process sequence, the highly-purified alginate is washed in ethanol and, if appropriate, then washed in water and air-dried at room temperature.

The purified alginate has, as a function of the source material, a ratio of the monomer mannuronic acid and guluronic acid in the range from 0.1 - 9 (corresponding to 1 % to 90 % mannuronic acid) and a mean molecular weight from approx. 10 kD to more than 1000 kD. Alginate purified in this way, implanted in an autoimmune diabetic BB/OK rat, does not incur any foreign body reaction after an implantation period of 3 weeks, or only a negligibly small reaction, as is explained in greater detail hereinafter.

The process described for the acquisition of alginate can be

modified with regard to the precipitation medium, the choice of the sedimentation medium, the choice of the inert propellant gas, the precipitation process, and/or the procedure with the collection of the precipitated alginates. Instead of the diatomaceous earth used for the sedimentation, any other absorbent material can also be used, such as electrographite, granulate, cellulose, or porous recycling materials in powder or particle form. It is also possible to use stirring equipment coated with an absorbent material.

The object of the invention is also a highly-purified alginate which consists of a mixed polymer of mannuronic acid and guluronic acid in a proportion of the range from 1 % to 90 %, in particular about 70 %, whereby the mean molecular weight is greater than 1000 kD, but at least greater than 250 kD.

Alginates according to the invention feature characteristic substance properties, due to their extreme purity and gentle extraction technique with the process according to the invention, which are not found with conventional alginates. These substance properties comprise both characteristic parameters, which are directly measurable as properties of the alginate (e.g. viscosity), as well as parameters which, as properties of the impurities removed according to the invention, indicate the high purity of the alginate according to the invention due to their complete absence or their negligibly small presence (e.g. fluorescence properties of impurities, initiation of immunological reactions in animal experiments and in cell cultures, etc.).

An alginate according to the invention is characterised by a high viscosity. An aqueous solution of an alginate according to the invention with a 0.1 % concentration has a viscosity in the range from 10 to 15 mPa s. This represents a substantially higher value in relation to the viscosity of conventional alginate solutions of the same concentration (about 1 to 5 mPa s). With 0.5 % solutions of the alginates according to the invention, a viscosity is derived of 280 mPa s. The viscosity of

the alginate solutions is determined with a ball viscosimeter (type AMV-200, Anton Paar KG, Fraz, Austria). The molecular weight is determined from the viscosity values by means of the process according to Huggins ("J. Am. Chem. Soc.", Vol. 64, 1942, p. 2716 ff.) and Krämer ("Ind. Eng. Chem.", Vol. 30, 1938, p. 1200 ff.). With the alginates according to the invention, mean molecular weights are derived which are larger than 250 kD.

Alginates according to the invention are free of phenols and compounds similar to phenols, in particular of polyphenols, which are composed of phloroglucinol, and phenol-protein compounds. With an excitation wavelength of 366 nm, alginates according to the invention, apart from a solvent fluorescence (Raman bands of the water) at 418 nm in the spectral range from 380 to 550 nm, do not have any fluorescence emissions. The freedom from phenols and polyphenols of the alginates according to the invention is also derived from colour tests with the use of the Folin-Denis reagent or with dimethoxybenzaldehyde (DMBA). Alginates according to the invention do not have any extinction during these colour tests, apart from the solvent media absorption.

Alginates according to the invention are practically free of substances (e.g. proteins) which absorb at a wavelength of 350 nm. This freedom from proteins is in turn manifested in a fluorescence measurement with an excitation wavelength of 270 nm, which, apart from the solvent medium fluorescence, does not emit any fluorescence in the range from 300 to 500 nm. The freedom from proteins of the alginates according to the invention is also manifested with the photometric proof of protein according to Bradford ("Anal. Biochem.", Vol. 72, 1976, p. 248 ff.).

Alginates according to the invention are also endotoxin-free. Endotoxins are impurities which, on implantation, may incur an immune reaction by the recipient, which also passes out of the cell walls of the bacterial concomitant flora of the alginates into conventional algae extracts. Alginate solutions according

to the invention (concentration: 0.25 %) have an endotoxin content of less than 14.5 endotoxin units per millilitre of alginate solution (measuring process: Quantitative endotoxin determination with Limulus amoebocytes Lysate test).

5

Alginates according to the invention are biocompatible, by contrast with conventional alginate extracts, inasmuch as this is demonstrated by the XTT and MTT tests explained hereinafter and by experiments on BB/OK rats.

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Preferred uses of a highly-purified alginate of this nature are transplant surgery, in which living cells are enclosed in an alginate capsule, and are implanted in the body of a living being without the initiation of immunological reactions. The highly-purified alginate can also be used in the food or textile technology sectors for increasing the compatibility of certain specific foodstuffs or substances. It is emphasised that the process according to the invention as explained heretofore is also well-suited for the manufacture of highly-purified alginate with lower molecular weight, up to 1000 kD.

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Further advantages and details of the invention are described hereinafter, in particular with reference to the appended figures. These show:

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Fig. 1      A sectional view of a nozzle head for the manufacture of alginate capsules;

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Fig. 2      Fluorescence spectra for the demonstration of the freedom from phenol of the alginates according to the invention;

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Fig. 3      Fluorescence spectra for the demonstration of the freedom from protein of the alginates according to the invention;

Fig. 4      Results of a lymphocyte simulation test for the

demonstration of freedom from immunogens of alginates according to the invention.

Examples of performance

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Hereinafter specific embodiments of the procedure explained heretofore are described, on the basis of examples. In this situation, reference is made without restriction to the purification or use of alginate material on the basis of brown  
10 algae. Instead of brown algae, in general all salt-water or fresh-water algae containing alginate can be used. The purification of alginate material from other algae is effected in the corresponding manner.

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The source material is comprised of (1) fresh brown algae, (2) dried brown algae, or (3) commercial alginate. As fresh material (1), brown algae harvested in nature or in a cultivation area or greenhouse, or brown algae portions from predetermined development stages of the life cycle of the algae or  
20 corresponding algae material cultivated in a homogenous bioreactor are used. The drying material (2) consists of dry brown algae, acquired according to these alternatives.

20

Example 1

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In the first example, reference is made to 80 g dry and then rehydrated (watered) algae. With commercial alginate, of which the dry weight makes up only about 1 % of the fresh weight, correspondingly less dry weight is used. The dry material (e.g.  
30 Laminariales, Fucales) according to (2) or (3) are watered for several hours in hot mains water, in accordance with the initial quantity. This may last, for example, for at least 3 to 4 hours with mains water with a temperature of 40 °C, whereby the water is replaced several times, or flows. With the use of cold water,  
35 the watering must be extended accordingly. Watering is effected for preference by the material being suspended in a water-permeable container (e.g. water-permeable bag) in flowing mains water. With watering in standing water, the material is, for

30

35



example, watered in 3 to 6 litres of water at an initial dry weight of about 80 g of dry algae (corresponding to 10 % of the fresh weight of about 800 g). After watering, the procedure takes place in an analogous manner for all three types of source material (1) to (3) mentioned.

The material is suspended in about 5.7 litres of a 23 mN EDTA solution (distilled or desalinated water) or, in the case of commercial alginate (3), is dissolved. The effect of the ethylene diamine tetra acetic acid (EDTA) solution takes place for at least 10 hours. The effect time can be shortened if the suspension is constantly stirred. As the effect time increases, so the yield of purified alginate improves. With an unstirred suspension, for example, an effect time of several days can be provided for.

5 %  $\text{Na}_2\text{CO}_3$  and EDTA as solid substances are now added, under stirring. The EDTA quantity is selected in such a way that a 50 mN EDTA solution is formed. The suspension is stirred until a homogenous (fine dispersed) solution is achieved. This state is achieved in particular if only a few cell constituents are still visible in the solution. Some 34 g diatomaceous earth is then added under stirring, and the solution is stirred for at least 2 days. It is possible, as an alternative, for the diatomaceous earth to be added together with  $\text{Na}_2\text{CO}_3$  and EDTA under stirring. Provision can be made, depending on the application (in particular as a function of the brown algae material used), for ion exchange material additionally to be added simultaneously with the diatomaceous earth or electrographite. It is possible, for example, for 34.2 g amberlite to be added as an ion exchanger, which has been subjected to purification beforehand. This purification serves to remove toxic substances and comprises watering in flowing water (duration about 3 hours).

After the diatomaceous earth treatment, the volume of the solution is diluted to 22.8 l with desalinated water, in order to reduce the viscosity. The dilution is selected in general in such a way that the solution is subsequent capable of being

filtered. The addition of water accordingly depends in particular on the brown algae material used. After the dilution and brief stirring, the solution is allowed to stand for at least 10 hours, in order for solid constituents to sediment out.

5 The standing time may be of the order of days. The supernatant is decanted off and filtered. The filtration takes place in multiple stages, whereby initially a deep filter with a pore size of 15  $\mu\text{m}$  and then a filter with a pore size of 0.1  $\mu\text{m}$  is used.

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The addition of salt to the filtrate then follows. The addition of KCl is preferred, whereby, depending on the application, other appropriate salts may also be added. So much KCl is added as solid substance as will produce 0.13 M KCl solution.

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20 An ethanol precipitation follows. The quantity of ethanol added depends on how much fucoidan is present in the material. With the first ethanol precipitation, the ethanol concentration should not exceed 40 % in the presence of fucoidan, since otherwise the fucoidan will precipitate in sympathy. In the example given, about 12 litres of 99 % ethanol is added, so that the final concentration of ethanol in the total solution amounts to about 34 %. Provision can also be made for the ethanol concentration to be selected as a function of the nature of the precipitation of the alginate. By variation of the ethanol concentration, the result can be achieved that the alginate precipitates in thread form or in wad form. The attempt is made to achieve such a precipitation as far as possible, in order for the alginate to float and is capable of undergoing further processing, as explained hereinafter.

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Instead of ethanol, at least one other alcohol (e.g. isopropanol) or a precipitation acid can be added, whereby the concentration is based on the criteria indicated heretofore.

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During the precipitation process, a propellant gas flows through the solution (e.g. air). The precipitated alginate is driven upwards by the air being blown in, i.e. while it is being

formed, and can be easily lifted from the surface of the solution by a suitable device (e.g. net, sieve, or the like). The precipitated alginate can also be collected without the addition of propellant gas by decanting or stirring with a stirring device, remaining adherent to the precipitated alginate. The collected alginate is then dewatered with a filter press, in order to remove the water at least in part from the strongly hygroscopic material.

10 The procedural stage carried out up to this point are repeated, depending on the application, in full, in part, or in part under modified conditions. Further processing of the precipitated alginate is then carried out as follows.

15 The precipitated alginate is dissolved in 11.4 l and 0.5 M-KCl/10 mM-EDTA solution. Dissolving takes place under stirring, until a homogenous solution is achieved. A second precipitation then takes place with about 10 litres of a 99 % ethanol solution under the introduction of propellant gas. Under these conditions the alcohol concentration in the total solution amounts to about 20 44 %. For this second precipitation, a higher alcohol (or acid) concentration can be selected, since the fucoidan (see above) is already separated. The concentration of the precipitation medium is again adjusted in such a way, however, that the formation of 25 wad-type alginate is promoted. With an alcohol concentration which is not optimum, the alginate is of the nature of gelatine and therefore cannot float (floating in the surface under the effect of propellant gas).

30 The precipitated alginate is then again dewatered through a filter press and washed several times with ten times the quantity of a 70 % ethanol solution. The material is then dried at room temperature. The drying can take place under sterile conditions. After the washing with ethanol, provision can be 35 made for the material to be washed several times with desalinated water or dialysed against desalinated water, in order to remove residual traces of concomitant substances.

The number of precipitations carried out according to the invention is based on the impurities or the toxic byproducts in the source material.

- 5 The tissue compatibility of the alginate acquired according to the Example is tested as follows. Implantation experiments are carried out with normoglycaemic (6.1 + 0.4 mM plasma glucose), diabetes-prone BB/OK rates (200 + 25 days old). These rats have a substantially higher sensitivity to impurities in alginates  
10 than the Lewis rats referred to earlier.

15 Ba<sup>2+</sup> alginate capsules were manufactured from the highly-purified alginate in accordance with the process described in DE-OS 42 04 012 A1. The alginate capsules have a mean diameter of 200 µm to 400 µm. Implantation was effected beneath the kidney capsule of the BB/OK rats. The recipient animals remained normoglycaemic and showed no loss in body weight. The animals were killed after three weeks, the kidneys removed, and fixed in Bouin's solution. After embedding in paraffin, the preparation of 7 µm sections  
20 was carried out. Every 20th section from two independent individuals were taken for histological examination.

The result of the investigation for different samples in comparison with raw alginate is compiled in the following table:

25 Result for two independent samples:

Sample	Rat #	Histological evaluation	Endotoxin content of a 0.25 % solution of the alginate
S1	V159	(+)	1 EU/ml
30	V161	+	
S2	V454	0	3.5 EU/ml
Raw alginate	V12	++++	> 1000 EU/ml

(Legend:

- 35 0: No reaction, (+): very weak reaction, +: weak reaction, ++++ very strong fibrosis)

It can be seen that the implantation with the alginate according

to the invention does not cause any reaction, or only a very weak one, while by contrast implantation with commercially available raw alginate incurs a very strong fibrosis.

- 5 The endotoxin content, which is characteristic of a potential incidence of bacteria, shows excellent values in the case of highly-purified alginates, values which are virtually negligible, while by contrast the comparison value of commercial raw alginate is about a thousand times greater.

- 10 It was determined with a highly-purified alginate according to the invention, with the use of the following test procedures, that no impurities of toxic substances were incurred. The test procedures comprise in particular fluorescence spectroscopic  
15 processes and endotoxin or mitogen activity essays, such as are described in G. Klock et al. in "Appl. Microbio. Biotechnology" (Vol. 40, 1994, page 638 ff.) and in "Biomaterials" (Vol. 18, 1997, page 707), NMR-spectroscopic processes, the determination of the antioxidative potential of the highly-purified alginate  
20 by determination of the reaction to the addition of HOCl, and by the determination of the oxidative activity of neutrophilic granolycytene, with the aid of chemiluminescence (see K. Arnold in "Proceedings of the Saxony Academy of Sciences in Leipzig", Vol. 58, 1997, Book 5) and the process of "Free Flow  
25 Electrophoresis", as described by U. Zimmermann et al. in "Electrophoresis" (Vol. 13, 1992, page 269). No toxic contamination was determined with this process.

- 30 According to the process described in the publication referred to above by G. Klöck et al. (1997), in addition, the proportion of mannuronic to guluronic acid was determined with the aid of what is referred to as "Circular Dichroismus-Spectroscopy" or with IR spectroscopy. In addition, the molecular weight was  
35 determined by the analysis of the viscosity.

The capsules of alginate cross-linked with  $Ba^{2+}$  possess excellent elasticity, as was proved with the aid of compression measurements.

## Example 2

With the second example, 10 g dry algae was taken as the reference. With larger initial quantities, the quantitative sizes given hereinafter are to be converted accordingly in linear manner. As a departure from Example 1, the acquisition and purification of alginate is effected to advantage without watering. The dry source material is, instead, added directly into the EDTA solution. The EDTA solution has a concentration in the range from about 10 to 50 mM. The suspension is stirred for 24 hours and then screened to remove solid material. A further advantage of the process explained according to the second Example is that the EDTA consumption, and also the use of alcohol, is reduced.

Provision can be made for additional activated carbon to be added to the suspension during the EDTA treatment. The amount of the activated carbon added amounts for preference to some 10 to 200 % of the dry algae mass which is weighed in.

This is followed immediately and without a separate sedimentation stage by a filtration of the suspension. The filtration takes place in two stages, whereby a pre-filter or deep-filter is used first, with a pore size of 15  $\mu\text{m}$ , and then a filter with a pore size of 0.2  $\mu\text{m}$ .

After the addition of salt to the filtrate as in Example 1 (e.g. formation of a 0.13 M KCl solution) several ethanol precipitations take place. For the first precipitation, 99 % ethanol (denatured with acetone) is added, with the result that a final concentration of the ethanol in the total solution of 37.5 % is attained. The precipitation product is collected and added to a 0.5 M KCl solution (without EDTA). The volume of the KCl solution is adjusted to a third of the solution volume prior to the precipitation.

For the second precipitation, 99 % ethanol (denaturised with

acetone) is added, so that a final concentration of ethanol in the total solution of 44 % is attained in this case. The precipitation product is collected and added to bi-distilled water with a volume corresponding to the volume of the KCl solution after the first precipitation.

Before the third precipitation a dialysis can be carried out of the precipitation product derived from the second precipitation. The dialysis, which is not a mandatory feature of the process, is effected for a period of three days, with a change of water three times per day. For the third precipitation, 99 % ethanol (denaturised with acetone) is again added to adjust a final concentration of the ethanol in the total solution of 50 %. The precipitation product is collected and dried.

### Example 3

An example for transplant surgery is described hereinafter, namely the micro-encapsulation of the Islets of Langerhans.

Isolated Islets of Langerhans were suspended in a solution of 0.9 % NaCl and 0.5 % of the purified alginate. This suspension was introduced in fine drop form through a spray nozzle shown in Fig. 1. The nozzle has a movable inner hollow tube (inner nozzle) with a lur connection (inner diameter 350  $\mu$ m or 2 mm), a middle hollow tube (middle nozzle) with a diameter of 1 mm or 3.5 mm respectively and an outer channel 40, which opens into an adjustable air focusing head 50. These elements were fitted to a nozzle head 60, at which compressed air and lur connections are located.

The Islet suspension in alginate is pressured through the central nozzle channel. A solution of 0.5 to 2 % of the alginate in a 0.9 % common salt solution is applied through the surrounding channel. With the drops which occur at the nozzle outlet in this way, the outer alginate solution (0.5 to 2 %) surrounds the islets suspended in the 0.5 % alginate solution. Compressed air is introduced through the outer, third channel,

which shears the drops off from the nozzle aperture. The compressed air was adjusted to 30 to 40 mbar (7 to 8 l/min). The alginate drops 70 were delivered in 40 mL cross-linking solution with 20 mM BaCl<sub>2</sub> and 5 mM histidine. The cross-linking solution is adjusted with NaCl to a physiological concentration (290 mOsmol). The capsules were then washed accordingly three times with isotonic common salt solution.

With the alginate according to the invention, general encapsulations can be created for allogenic and xenogenic tissues, and for endocrinic tissues in particular.

Further characterisation of the alginate material according to the invention

1. Fluorometric test of phenol

Alginates according to the invention do not contain any contaminating impurities based on phenols, polyphenols, or other phenol compounds. This freedom from phenols means that the impurities referred to are not contained in the alginates, or in such a small volume that applications in biology and medicine, and the applications referred to heretofore in particular, will not be interfered with by immune reactions or the like. The freedom from phenol is demonstrated with a fluorometric analysis, as described by G. Skajk-Braek et al. (see "Biotechnology and Bioengineering", Vol. 33, 1989, p. 90 ff.). The measurement of the fluorescence is carried out with an LS50 spectrometer (Perkin-Elmer, Beaconsfield). At an excitation wavelength of 366 nm, the fluorescence spectra in solution samples, as shown in Fig. 2, are derived during the purification according to Example 2. Before and after the initial filtration, the alginate solution shows a strong fluorescence in the range between 380 and 550 nm (upper spectra). After the filtration and precipitation stages, the fluorescence is substantially decreased. The concentration of the final solution amounts to about 0.2 - 0.3 %. There remains only a fluorescence maximum at 418 nm, which corresponds to the solvent medium fluorescence. In



the alginate purified according to the invention, the fluorescence of the phenols and phenol compounds is reduced to less than about 10 % in relation to the unpurified alginate solution.

Fig. 2 shows that the phenol contents decrease drastically in the course of the process according to the invention, and that the purified alginate shows an emission which is only slightly above the values of highly-purified water.

## 2. Fluorometric test of protein

Freedom from protein of the alginate according to the invention is demonstrated as with the fluorometric analysis according to 1. Fig. 3 shows that, with an excitation wavelength of 270 nm before purification, a strong emission is measured in the range from 300 to 500 nm. This emission falls in the course of the purification to a value below 20 % of the fluorescence of the unpurified alginate solution. After the last precipitation, fluorescence is no longer demonstrable, or is negligibly small.

## 3. Test of phenol according to Folin-Denis or with DMBA

The Folin-Denis proof colours all compounds which contain phenol. Alginates manufactured according to the invention do not show extinction under this test. The Folin-Denis test is carried out under the following conditions:

The alginate solution which is to be analyzed (0.5 ml) is mixed with 1 ml methanol for extraction. The specimen is placed in the refrigerator after intensive shaking for about 8 hours. The precipitated alginate is centrifuged off, and 10 µl of the residue is added to 40 µl of the Folin-Denis reagent (Fluka, Deisenhofen, Germany), 80 µl 1 M Na<sub>2</sub>CO<sub>3</sub> and 120 µl H<sub>2</sub>O (sequence maintained). After intensive shaking, the colour development takes place in the water bath at 50 °C for 30 minutes. Measurement of the optical density follows with the aid of a photometer at a wavelength of 650 nm (or 725 nm).

With the addition of dimethoxybenzaldehyde (DMBA) too, the alginates according to the invention do not show any photometrically assessable colour reaction. This test was  
5 conducted under the following conditions: The alginate solution to be analyzed (0.5 ml) is mixed with 1 ml methanol for the extraction. The sample is placed in the refrigerator after intensive shaking for about 8 hours. The precipitated alginate is centrifuged off. This is followed by the addition of 10 µl of  
10 the residue into the DMBA solution, which is manufactured as follows: 2 g DMBA (Fluka, Deisenhofen, Germany) is dissolved in 100 ml glacial acetic acid. 16 ml HCl (37 %) is topped up to 100 ml with glacial acetic acid. Both solutions are to be mixed shortly before use, 1:1 to form the working reagent.

15 Measurement of the optical density then follows with the aid of a photometer at a wavelength of 490 nm (or 510 nm) against a calibration series of phloroglucinol (Sigma, Deisenhofen, Germany).

#### 20 4. Protein test according to Bradford

The proof of protein according to Bradford (see "Anal. Biochem.", Vol. 72, 1976, p. 248 ff.) is based on the fact that  
25 the colouring agent Coomassie brilliant blue bonds very specifically to proteins, as a result of which a colour break from red to blue takes place. The intensity of the blue colouring is in linear correlation with the amount of the protein, and can be quantified photometrically. With this  
30 method, proteins can be demonstrated into the lower µg range. 10 µl of a 0.5 % alginate solution was examined. The proof substance containing the colouring agent added to the alginate solution was what is known as the Bradford Reagent (manufacturer: Sigma, Steinheim, Germany). After an incubation  
35 period of 10 minutes, the sample absorption was measured photometrically at 570 nm, with the use of a "Thermomex Microplate Reader" device (manufacturer: Molecular Devices, Manlow Park, USA). No proteins were found in the alginate

solution according to the invention with this process.

## 5. XTT and MTT tests

- 5 The biocompatibility of the alginates purified according to the invention can be demonstrated with the following tests. Live cells, such as mouse lymphocytes or fibroblasts are kept in culture with the alginates for a few days. If the alginates demonstrate an immunogen effect, the cells will be stimulated or  
10 inhibited (in order to make the effect of the stimulation clearer, additional stimulants, such as LPS or PHA, are added to the culture). The metabolic activity, increased with lymphocytes and decreased with fibroblasts, can be visualised on the basis of the conversion of a colouring agent (XTT or MTT) by  
15 mitochondrial dehydrogenases and quantified photometrically. With alginates purified according to the invention, the colouring agent conversion is negligibly small (see Fig. 4), while with commercial alginates, by contrast, a sharp colouring agent conversion can be demonstrated. Fig. 4 shows the results  
20 of the colour test for various different LPS pre-stimulations.

- The cultivation of the cells was carried out under the following conditions. The lymphocyte cell suspension ( $1 \cdot 10^6$  cells/ml in Complete Growth Medium) is mixed with various different  
25 concentrations of lipopolysaccharides (LPS, final concentration: 0.01  $\mu\text{g/ml}$ ) and alginate solution (final concentration: 0.05 %), and cultivated for three days at 5 %  $\text{CO}_2$  and 37 °C. Thereafter the suspension is incubated with a solution of tetrazolium salt (XTT or MTT), and for a further 6 hours. This is followed by the  
30 measurement of the optical density at 450 nm (reference measurement at 650 nm).

## 6. Experiments in BB/OK rats

- 35 As described heretofore for Example 1, the biocompatibility of alginates according to the invention can also be demonstrated by in-vivo tests on BB/OK rats, the immune system of which features an increased macrophage activity. Alginate purified according to

the invention, implanted in accordance with the steps described in capsule form in rats' kidneys, did not cause any fibrosis after an incubation period of three weeks.

## 5 7. Electrorotation

10 The biocompatibility of the alginates purified according to the invention can also be demonstrated with the following test. Live cells, such as human lymphocytes or mouse lymphocytes, are kept in culture with alginates for some days. If the alginates shown an immunogen effect, the cells react with a substantially increased membrane surface (increase in the microvilli; to make this effect clear, additional stimulants such as LPS or PHA may be added to the culture). Such an increase can be determined by means of an increase in the specific membrane capacity  $C_m$ . The electrorotation of the cells under the effect of high-frequency electric fields offers a possibility of demonstrating these changes in the lymphocyte membrane on the level of the individual cell. With alginates purified according to the invention, no increase in the specific membrane capacity can be measured, while by contrast with commercial alginates an increase in this value can be demonstrated.

25 The measurements were carried out under the following conditions. The lymphocyte cell suspension ( $1 \cdot 10^6$  cells/ml in Complete Growth Medium) is mixed with an alginate solution (final concentration: 0.05 %) and cultivated for three days at 5 %  $CO_2$  and 37 °C. Thereafter the suspension is washed twice in isoosmolar inositol solution (290 mOsm/kg), then the suspension is thoroughly repeated. With different conductivity values (10, 25, 40  $\mu S/cm$ ), adjusted with HEPES-KOH, pH 7.2) the determination of the characteristic frequency (maximum) of the antifield rotation is carried out by the compensation method. With the aid of a linear regression of the characteristic frequencies, normed with the cell radius, applied against the external conductivity, the electrical parameters (capacitance and conductivity) of the plasma membrane can be determined. The electrical parameters of the cells cultivated in the presence of

the alginates according to the invention remain unchanged, while by contrast the cells cultivated with commercial alginates feature an increase in membrane capacity by 10 to 50 %.

5 8. Cell size and cell number

10 The biocompatibility of the alginates purified according to the invention can also be demonstrated with the following test. Live cells, such as human lymphocytes or mouse lymphocytes, are kept in culture with alginates for some days. If the alginates feature an immunogenic effect, the cells will increase in size and the number of cells will increase likewise (in order to make this effect clear, additional stimulants, such as LPS or PHA, can be added to the culture). The cell size and the number of  
15 cells can be determined with the aid of the CASY1 Cell Analyzer (model TTC, Schärfe Technology, Reutlingen, Germany), which works on the Coulter principle. Such a device allows for the rapid and precise analysis (size, size distribution, and cell number) of several thousand cells per measurement. With  
20 alginates purified according to the invention, no significant cell enlargement or increase in cell numbers can be measured after incubation, while with commercial alginates, by contrast, a clear cell enlargement (about 50 %) and an increase in the number of cells (35 to 50 %) can be demonstrated.

25

The measurements are carried out under the following conditions. The lymphocyte suspension ( $1 \cdot 10^6$  cells/ml in Complete Growth Medium) is mixed with an alginate solution (final concentration: 0.05 %) and cultivated for three days at 5 %  $\text{CO}_2$  and 37 °C.

30

Thereafter an aliquot (100  $\mu\text{l}$ ), centrifuged off and absorbed in inosit (or PBS) of the lymphocyte suspension, after thorough repeat suspension, is washed twice in isoosmolar inositol solution (290 mOsm/kg), and absorbed in 10 ml PBS (phosphate-buffered saline) and measured immediately in the CASY1. From the  
35 CASY1 histogram, the proportion of the coarse, stimulated cells in the total population can be calculated.

## CLAIMS

1. Process for obtaining a highly-purified alginate composition, with the stages:
  - Extraction of algae material or raw alginate in a solution with a complex forming agent,
  - Filtering of the solution,
  - Precipitation of the alginate out of the solution, and
  - Collection of the precipitated alginate.
2. Process according to Claim 1, in which ethylene diamine tetra acetic acid is used for the extraction as a complex forming agent.
3. Process according to Claim 1, in which the extraction takes place in a soda solution.
4. Process according to Claim 2 or 3, in which activated carbon is added for the extraction of the solution.
5. Process according to one of the foregoing Claims, in which, before the filtering of the solution, sedimentation of cell constituents and particles is carried out with a porous binding agent from the solution.
6. Process according to Claim 5, in which the sedimentation takes place with a porous granulate on the basis of diatomaceous earth, cellulose, or recycling materials from regenerated raw materials.
7. Process according to one of the foregoing Claims, in which the filtering takes place with deep filters of decreasing pore size in each case.
8. Process according to one of the foregoing Claims, in which the precipitation of the alginate takes place with ethanol.
9. Process according to Claim 6, in which the ethanol content

is selected in the range from 10 - 50 %.

- 5 10. Process according to one of the foregoing Claims, in which the collection of the precipitated alginate is effected by foaming out of the solution, by decanting the solution, or by stirring the solution with a stirring and collecting device.
- 10 11. Process according to one of the foregoing Claims, in which the dewatering of the alginate takes place at room temperature.
- 15 12. Process according to one of the foregoing Claims, in which, after the dewatering, the extraction, filtering, precipitation and dewatering are repeated at least once again.
- 20 13. Process according to one of the foregoing Claims, in which use is made as the algae material of fresh algae material occurring in nature or fresh algae material cultivated in a bioreactor or tank system, or algae material from fused or regenerated algae cells or commercial alginate.
- 25 14. Process according to one of the foregoing Claims, in which use is made as the algae material of specific organ or tissue parts of algae or algae parts, or specific organ or tissue parts of algae or algae parts from specific stages of the development cycle of algae.
- 30 15. Process according to one of the foregoing Claims, in which use is made as the algae material of brown algae or other alginate-producing fresh-water or salt-water algae.
- 35 16. Alginate composition, which as a mixed polymer consists of mannuronic acid and guluronic acid,  
**characterised in that,** in the mixed polymer, a ratio pertains of mannuronic acid to guluronic acid in the range from 1 % to 90 %, and the mean molecular weight of the

mixed polymer is greater than 250 kD.

17. Alginate composition, which in an aqueous solution with a 0.1 % concentration, has a viscosity in the range from 10 to 15 mPa . s.
18. Alginate composition, which in an aqueous solution with a 0.5 % concentration, has a viscosity in the range from 250 to 300 mPa . s.
19. Alginate composition, which in an aqueous solution and under illumination with an excitation wavelength of 366 nm in the spectral range from 380 to 550 nm, does not show any fluorescence emission.
20. Alginate composition, which under colour tests with the Folin-Denis reagent or with dimethocybenzaldehyde, does not show any colouration.
21. Alginate composition, which in an aqueous solution and under illumination with an excitation wavelength of 270 nm in the spectral range from 300 to 500 nm, does not show any fluorescence emission.
22. Alginate composition, which does not contain any proteins detectable with the photometric protein test according to Bradford.
23. Alginate composition, which when implanted in the kidneys of BB/OK rats, does not initiate any significant immunological reaction.
24. Alginate composition, which, according to the application of the XTT/MTT test, or the cell rotation method, or an electrical cell number and cell size analysis, does not lead to any detectable lymphocyte activation.
25. Alginate composition according to one of Claims 16 to 24,



26. Use of an alginate composition according to one of Claims  
5 16 to 25, for the manufacture of alginate capsules or  
coatings for transplant surgery and for other medical  
applications and for the food industry.

10

15

ABSTRACT

- 5 A process for obtaining a highly-purified alginate compound  
contains the steps of extraction of algae material or raw  
alginate with a complex forming agent in a solution,  
sedimentation of cell constituents and particles from the  
solution, filtering of the solution, precipitation of alginate  
10 from the solution, and collection of the precipitated alginate.  
An alginate composition pertaining as a mixed polymer of  
mannuronic acid and guluronic acid contains a ratio of  
mannuronic acid to guluronic acid in the range from 1 % to 90 %  
and a mean molecular weight of up to or above 1,000 kD.  
15 (Fig. 2)

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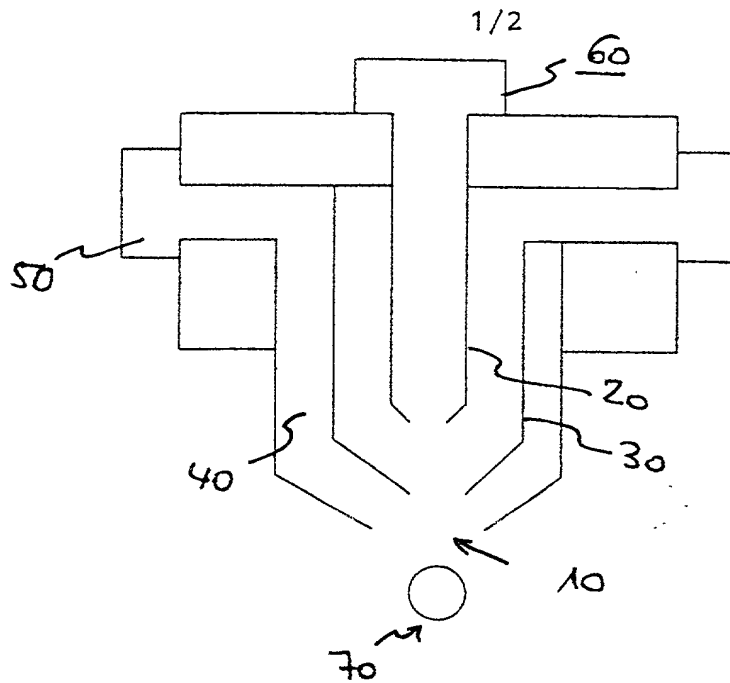
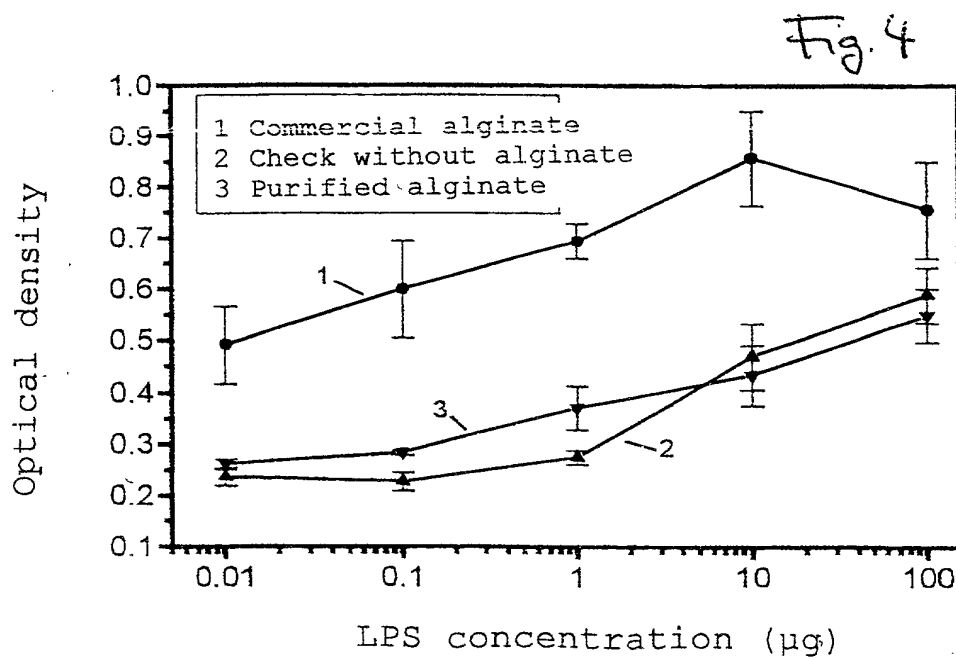


Fig. 1



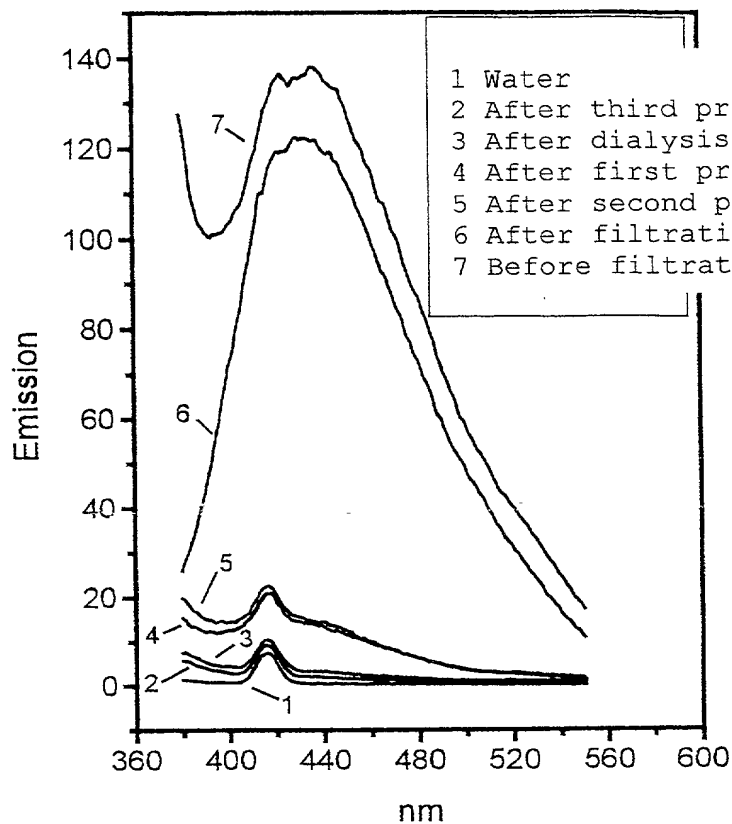


Fig. 2

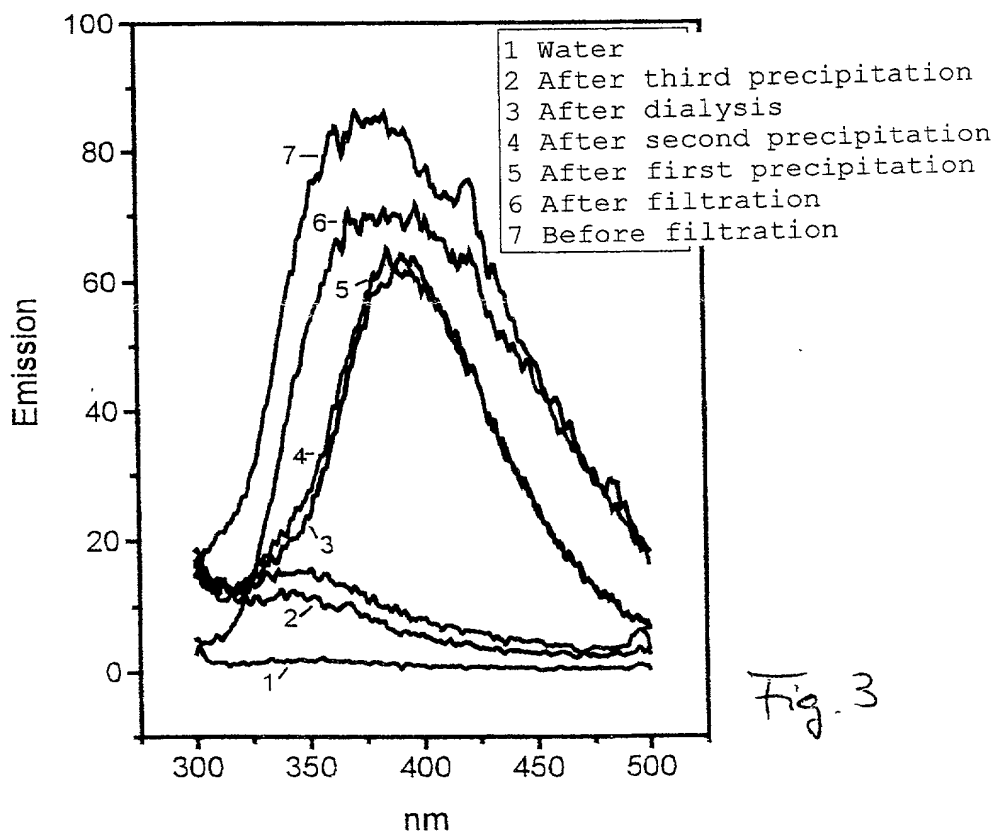


Fig. 3

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#3

**COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: PROCESS FOR OBTAINING HIGHLY-PURIFIED ALGINATES the specification of which:

(check one) ☐ is attached hereto.

☒ was filed on 02/13/2001 as Application Serial No. 09/762,850

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application (s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claim:

Prior Foreign Application(s)

Priority Claimed

19836960.3    Germany    14 / 08 / 98  
(Number)    (Country)    D/M/YR Filed

☒ YES    ☐ NO

PCT/EP99/05987    PCT    12 / 08 / 99  
(Number)    (Country)    D/M/YR Filed

☒ YES    ☐ NO

\_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_  
(Number)    (Country)    D/M/YR Filed

☐ YES    ☐ NO

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application (s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the

first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

(Application Serial No.)	(Filing Date)	(Status)
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### POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorneys jointly and each of them severally, with full power of substitution, delegation, and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith:

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## DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## SIGNATURES

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Inventor's signature 

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